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14. ABSTRACT A major challenge in prostate cancer research is to develop novel therapies that can delay or prevent the progression of androgen-sensitive prostate cancer to androgen-independence. Androgen receptor (AR) is often overexpressed and plays an essential role in androgen-refractory prostate tumors. Our preliminary studies suggest that heat shock protein 90 (Hsp90) is required for androgen-independent AR nuclear localization in androgen-refractory prostate cancer cells. This project will test our research hypothesis that Hsp90 plays a critical role in ligand-independent AR nuclear localization, an essential step leading to androgen-refractory prostate cancer. In the last year of the funding period, we have constructed lenti-viral vectors for expressing siRNA specific for Hsp90. Experiments are on the way to test if down-regulation of Hsp90 protein will cause alterations in AR intracellular localization and function in prostate cancer cells.					
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Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4-7
Key Research Accomplishments.....	7
Reportable Outcomes.....	8
Conclusion.....	8
References.....	8
Appendices.....	8

Introduction

Prostate cancer (CaP) is the most common cancer and the second most common cause of cancer death among men in the United States (1). The androgen deprivation therapy (ADT) is the standard treatment for metastatic CaP. However, patients invariably recur with hormone-refractory or androgen-independent (A-I) CaPs. Effective treatment for A-I CaP is desperately needed for this lethal disease. Recent studies showed that, although the A-I tumors are androgen depletion independent, their growth is androgen receptor (AR)-dependent (2, 3). Novel strategies to inhibit ligand-independent AR activity may be effective against A-I prostate tumors. Androgen receptor (AR), a member of the steroid receptor superfamily, is a ligand-dependent transcription factor that controls the expression of androgen-responsive genes (4). Intracellular trafficking is an important mechanism in the regulation of many transcription factors, including AR. In order to access its target genes, a transcription factor requires localization to the nucleus. Likewise, retention of a transcription factor in the cytoplasm prevents its activity. Thus, a key regulatory step in the action of AR is its nuclear translocation. In androgen-sensitive cells, AR is localized to the cytoplasm in the absence of ligand. Upon addition of androgens, AR translocates to the nucleus and transactivates target genes. However, in A-I prostate cancer cells, AR remains in the nucleus even in the absence of androgen and transactivates androgen-responsive genes, leading to A-I growth of prostate tumors (2, 3). Our preliminary studies demonstrate that the Hsp90 inhibitor, 17-AAG, prevents A-I nuclear localization and activation of AR in the androgen-refractory C4-2 cells (5). This study will further determine the role of Hsp90 in ligand-independent AR nuclear localization and prostate cancer progression to A-I using the C4-2 androgen-refractory prostate cancer cell line as a model.

Body

Task 1: Determining the effect of HSP90 level on AR nuclear localization in the absence of androgen (months 1-18).

- A. Develop lenti-viral expression vector for HSP90 protein and siRNA specific for HSP90 (months 1-10)
- B. Infect LNCaP cells with HSP90 expression lenti-virus and assay for the effect of HSP90 overexpression on AR intracellular localization (months 10-18)
- C. Infect C4-2 cells with HSP90 siRNA lenti-virus to assay the effect of HSP90 downregulation on the intracellular localization of AR (months 10-18)

Lentiviral expression vectors are chosen to express Hsp90 protein and siRNA in this project because they offer high infection efficiency. We have purchased lentiviral vectors pCDH cDNA cloning vector (Cat# CD511A-1) and pSIH-H1 shRNA cloning vector (Cat# SI500A-1) from System Biosciences (SBI) (**Fig. 1**). Both vectors express green fluorescence protein (GFP), which allows us to monitor the infection efficiency conveniently. We have generated one lenti-viral vector that expresses Hsp90 protein and another one that expresses siRNA specific for Hsp90. Unfortunately, the lenti-viral Hsp90 protein expression vector has a stop codon in the Hsp90 open reading frame. We are in the process of correcting the mutation in the expression vector. We expect to obtain the correct Hsp90 expression vector very soon.

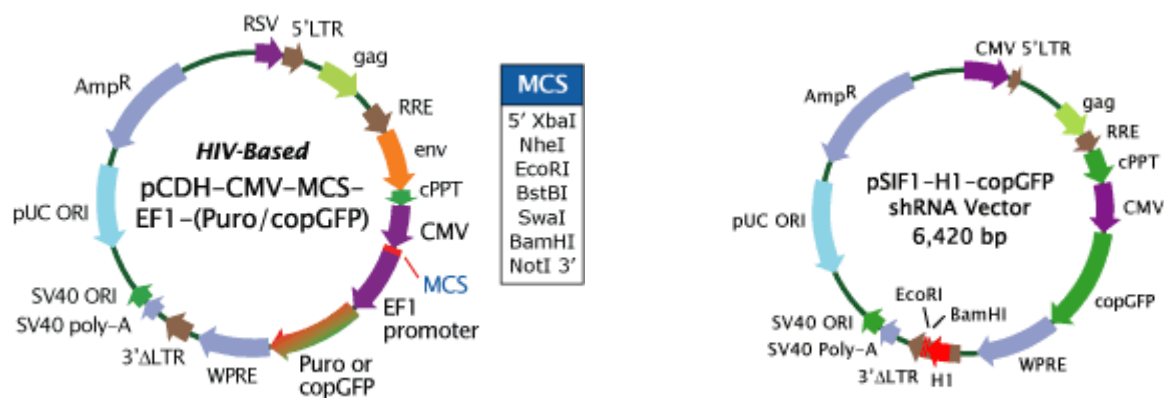


Fig. 1. Lenti-viral vectors. Left diagram shows the pCDH cDNA cloning vector (Cat# CD511A-1) for Hsp90 overexpression. This vector includes a CMV promoter before the multiple cloning sites and an EF1 promoter just before a green fluorescence protein (GFP) as a reporter for transduced cells. Right diagram shows the knockdown lentivector, pSIF1-H1 shRNA cloning vector (Cat# SI500A-1). This vector includes a CMV promoter for constitutive expression of GFP and an RNA polymerase III promoter to drive the expression of the siRNA sequence. Both vectors were purchased from System Biosciences (SBI) Mountain View California.

We have designed siRNA sequence using Integrated DNA Technologies RNAi online software tool (IDT, Iowa City, IA). The sequence of siRNA specific for Hsp90 is 5'GGACCAGGTAGCTAACTCA3'.

To generate Hsp90 expression vector, the human Hsp90 cDNA sequence was PCR amplified using forward (For) and reverse (Rev) primers with indicated sequences:

For: 5' CAGGCTAGCACCATGCCTGAGGAAACCCAGACCCA 3'

Rev: 5' TCAGGCGGCCGCTTAGTCTACTTCTTCCATGCG 3'.

The amplified cDNA was restriction digested and cloned into the multiple cloning sites (MCS).

Lentiviral vectors were next packaged into packaging cell line 293TN (SBI, California). An RSV-5LTR or CMV-5LTR is included in each of the vectors for expression of constructs into the producer cell line (293TN). The pPACK H1 plasmids: pPACKH1-GAG, pPACKH1-REV, and PVSV-G provide all the necessary elements to produce VSV-G pseudoviral particles. These particles provide the most efficient method for high titer transduction into target cells.

The siHsp90 construct was sequence verified and packaged. The pseudoviral particles were transduced into C4-2 prostate cancer cells. Based on GFP expression, about 60% of the cultured C4-2 cells were transduced by siHsp90 lenti-viral particles in our experiments. We have isolated siHsp90 vector transduced C4-2 cells by fluorescence activated cell sorting (FACS) analysis. **Fig. 2** shows that FACS sorted C4-2 cells are positive with GFP expression. These GFP-positive cells were used to determine if siHsp90 lenti-viral infection is effective in down-regulating Hsp90 expression. The data thus far are very promising.

The down-regulation of Hsp90 mRNA by siRNA specific for Hsp90 was revealed by real-time PCR (**Fig. 3**). The primer pair used for Real-time PCR are 5' TCTGGAAGATCCCCAGACAC 3' (Forward) and 5' AGTCATCCCTCAGCCAGAGA 3' (Reverse).

Down-regulation of Hsp90 protein by siRNA in C4-2 cells was reproducibly observed in Western blot analyses using anti-Hsp90 antibody (**Fig. 4**). This result indicates that our siRNA lenti-viral vector is effective in down-regulating Hsp90 protein expression.

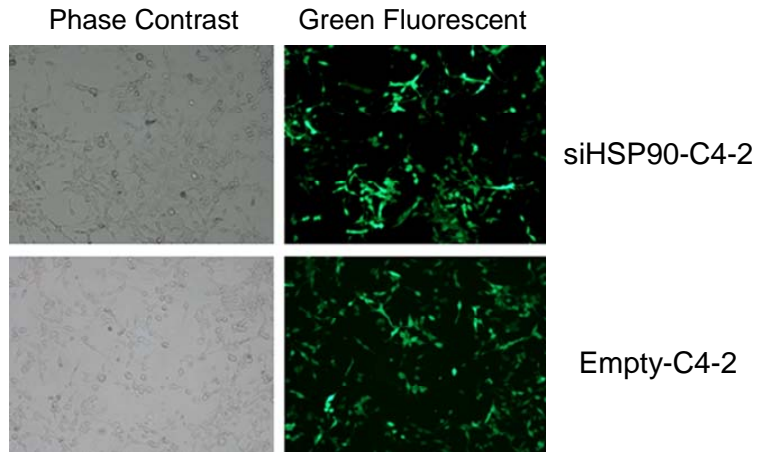


Fig. 2. C4-2 Cells Expressing GFP and siHsp90 Lentiviral Vector. Lentiviral Expression vector was packaged into 293TN cells. The viral particles were then used to infect parental C42 cells. Lentiviral vector was transduced and integrated into the genomic material of the parental cells. Cells expressing GFP include transduced lentivirus containing the sequence of interest. Since infections did not reaching 100% efficiency, the transduced cells were FACS sorted for GFP-positive cells.

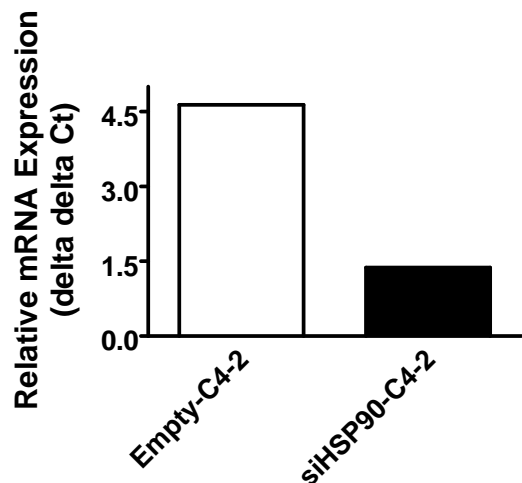


Fig. 3. Real-Time RT-PCR Analysis of Hsp90 mRNA expression in C4-2 Cells Transduced by Lentiviral siHsp90 Vector. Cells were harvested for at 90% confluency from a 6-well plate. RNA was isolated with Trizol® Reagent. cDNA synthesis and qPCR by Sybr Green were performed. The resultant graph is generated from one experiment done in triplicate. Expression of Hsp90 mRNA in siHSP90-C42 stable cells is knocked down by 3.4 fold relative to the empty vector infected control cells.

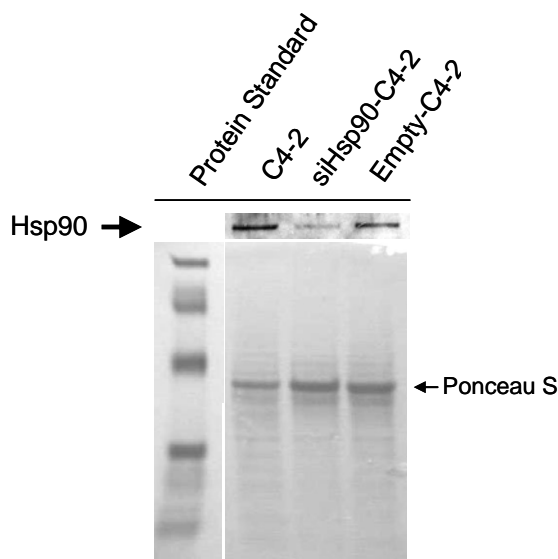


Fig. 4. Western Blot Analysis of Hsp90 Expression in C4-2 Cells Transduced by siHsp90 Lentiviral Vector. Cells were harvested in RIPA buffer containing protease inhibitors at ~90% confluency. Lysates were run on an SDS gel and transferred to membrane. Hsp90 was probed using anti-HSP90 $\alpha\beta$ (N-17). Empty vector infected C4-2 cells were used as a control in parallel. Parental C4-2 was also included in Western analysis.

Task 2: Test the hypothesis that HSP90 modulates the sensitivity of AR-positive xenograft tumors to androgen ablation (months 10-30)

We will shortly initiate studies in Task 2 using the lenti-viral expression vectors generated in Task 1.

Task 3: Determine the efficacy of HSP90 inhibitor in the inhibition of the prostate cancer progression to androgen-independence in LNCaP xenograft tumor model (months 20-36):

We will start Task 3 in the third year funding period.

Key Research Accomplishments

Construction of effective lenti-viral expression vectors is a prerequisite for testing our research hypothesis. We have successfully generated the proposed lenti-viral expression vectors. However, the efficiency of infection was about 60%. To obtain ~100% infected prostate cancer cells, we have sorted the infected cells by FACS and were able to purify infected cells. These sorted cells will allow us to accomplish Tasks 2 & 3 to determine the impact of Hsp90 up- and down-regulation on AR intracellular localization and androgen responsiveness of prostate cancer cells.

Reportable Outcomes

We have generated the proposed lenti-viral expression vector for siRNA specific for Hsp90.

Conclusion

Having generated the proposed lenti-viral expression vector, we are in the position to test our research hypothesis that Hsp90 plays a critical role in ligand-independent AR nuclear localization and progression of prostate cancer cells to androgen depletion independence.

References

1. Jemal, A., Murray, T., Ward, E., Samuels, A., Tiwari, R. C., Ghafoor, A., Feuer, E. J., and Thun, M. J. Cancer statistics, 2005. *CA Cancer J Clin*, 55: 10-30, 2005.
2. Chen, C. D., Welsbie, D. S., Tran, C., Baek, S. H., Chen, R., Vessella, R., Rosenfeld, M. G., and Sawyers, C. L. Molecular determinants of resistance to antiandrogen therapy. *Nat Med*, 10: 33-39, 2004.
3. Zegarra-Moro, O. L., Schmidt, L. J., Huang, H., and Tindall, D. J. Disruption of androgen receptor function inhibits proliferation of androgen-refractory prostate cancer cells. *Cancer Res*, 62: 1008-1013, 2002.
4. Zhou, Z., Wong, C., Sar, M., and Wilson, E. The androgen receptor: an overview. [Review]. *Recent Progress in Hormone Research*, 49: 249-274, 1994.
5. Saporita, A. J., Ai, J., and Wang, Z. The Hsp90 inhibitor, 17-AAG, prevents the ligand-independent nuclear localization of androgen receptor in refractory prostate cancer cells. *Prostate*, 67: 509-520, 2007.

Appendices

None.